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# Dissection of the relative contribution of the *Schizosaccharomyces pombe* Ctr4 and Ctr5 proteins to the copper transport and cell surface delivery functions

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The Ctr1 family of proteins mediates high-affinity copper (Cu) acquisition in eukaryotic organisms. In the fission yeast *Schizosaccharomyces pombe*, Cu uptake is carried out by a heteromeric complex formed by the Ctr4 and Ctr5 proteins. Unlike human and *Saccharomyces cerevisiae* Ctr1 proteins, Ctr4 and Ctr5 are unable to function independently in Cu acquisition. Instead, both proteins physically interact with each other to form a Ctr4–Ctr5 heteromeric complex, and are interdependent for secretion to the plasma membrane and Cu transport activity. In this study, we used *S. cerevisiae* mutants that are defective in high-affinity Cu uptake to dissect the relative contribution of Ctr4 and Ctr5 to the Cu transport function. Functional complementation and localization assays show that the conserved Met-$X_3$-Met motif in transmembrane domain 2 of the Ctr5 protein is dispensable for the functionality of the Ctr4–Ctr5 complex, whereas the Met-$X_3$-Met motif in the Ctr4 protein is essential for function and for localization of the hetero-complex to the plasma membrane. Moreover, Ctr4/Ctr5 chimeric proteins reveal unique properties found either in Ctr4 or in Ctr5, and are sufficient for Cu uptake on the cell surface of *Sch. pombe* cells. Functional chimeras contain the Ctr4 central and Ctr5 carboxyl-terminal domains (CTDs). We propose that the Ctr4 central domain mediates Cu transport in this hetero-complex, whereas the Ctr5 CTD functions in the regulation of trafficking of the Cu transport complex to the cell surface.

## INTRODUCTION

Copper (Cu) is an essential micronutrient for aerobic organisms, in which it plays several well-established roles as a structural and catalytic cofactor for a spectrum of proteins, including cytochrome *c* oxidase, Cu/zinc-superoxide dismutase (Cu/Zn-SOD), and multi-Cu ferroxidases involved in iron (Fe) uptake (Balamurugan & Schaffner, 2006; Kim *et al.*, 2008; Linder, 1991). However, when present in excess, the same redox properties that make Cu an indispensable element enable the generation of reactive oxygen species, such as hydroxyl radicals, which damage cells at the membrane lipid, protein and nucleic acid levels, and can interfere with the biogenesis of Fe–S clusters (Halliwell & Gutteridge, 1984; Macomber & Imlay, 2009). Therefore, micro-organisms must possess sophisticated mechanisms to tightly control the intracellular levels of Cu by regulating the transport of this metal across the plasma membrane.

A number of studies in *Saccharomyces cerevisiae* have allowed the identification of the proteins constituting the high-affinity Cu uptake system ($K_m$ around 1–5 μM). To be transported into *S. cerevisiae*, extracellular $Cu^{2+}$ is reduced to $Cu^{+}$ by the cell surface reductases Fre1 and Fre2 (Georgatsou *et al.*, 1997; Hassett & Kosman, 1995; Martins *et al.*, 1998), and is transported into cells by the functionally independent and biologically redundant plasma membrane Ctr1 and Ctr3 high-affinity Cu transport proteins (Dancis *et al.*, 1994b; Knight *et al.*, 1996; Peña *et al.*, 2000). The expression of Cu uptake genes, which include *FRE1*, *CTR1* and *CTR3*, is activated by the Mac1 transcription factor when environmental Cu becomes

Abbreviations: BCS, bathocuproine disulfonic acid; BPS, bathophenanthroline disulfonic acid; CTD, carboxyl-terminal domain; NTD, amino-terminal domain; SOD, superoxide dismutase; TMD, transmembrane domain.

A supplementary figure, showing that the $Met^{122}$ residue is essential for Ctr445 functionality, is available with the online version of this paper.

limited (Dancis *et al.*, 1994a; Georgatsou *et al.*, 1997; Knight *et al.*, 1996; Labbé *et al.*, 1997; Martins *et al.*, 1998). *S. cerevisiae ctr1*Δ*ctr3*Δ mutants exhibit Cu starvation phenotypes (Puig & Thiele, 2002), as shown by their inability to grow in media containing non-fermentable carbon sources such as glycerol and ethanol as the sole carbon source, given the significant loss of function of the Cu- and Fe-requiring mitochondrial cytochrome *c* oxidase. Furthermore, *ctr1*Δ*ctr3*Δ cells do not grow in media with limited Fe availability because of a defect in the Cu-dependent ferroxidase Fet3, which is essential for high-affinity Fe uptake in yeast, and they are highly sensitive to oxidative stress as a result of diminished Cu incorporation into Cu/Zn-SOD.

Topological studies *in vivo* have demonstrated that the *S. cerevisiae* Ctr1 Cu transporter contains three transmembrane domains (TMDs), with the amino-terminus localizing toward the extracellular space and the carboxyl-terminus facing the cytosol (Puig *et al.*, 2002). The extracellular amino-terminus of Ctr1 contains sequences rich in methionine residues that are arranged in Met-$X_2$-Met and Met-X-Met clusters, denoted Mets motifs. Genetic, biochemical and structural studies have revealed that the Mets motifs of Ctr1 are important for Cu acquisition when *S. cerevisiae* cells grow under Cu-deficient conditions, and that these motifs selectively bind $Cu^+$ ions through methionine thioester groups (Jiang *et al.*, 2005; Puig *et al.*, 2002; Rubino *et al.*, 2010). Indispensable residues for the function of Ctr1-mediated Cu transport include the last methionine ($Met^{127}$) of the Mets motifs, located at approximately 20 aa before TMD1, as well as the Met-$X_3$-Met motif in TMD2. The substitution of these conserved methionines with alanine or leucine completely abrogates Ctr1 function, without altering Ctr1 localization to the plasma membrane (Puig *et al.*, 2002). Interestingly, the substitution of these conserved methionines by cysteine or histidine does not eliminate Ctr1 transport activity, suggesting that these residues directly contribute to $Cu^+$ conduction through the plasma membrane toward the cytosol (Puig *et al.*, 2002). Genetic, biochemical and, more recently, structural data on the human Ctr1 protein indicate that Ctr1-like proteins oligomerize and assemble at the plasma membrane as symmetrical homotrimers with a cone-shaped pore in the centre (Aller & Unger, 2006; Dancis *et al.*, 1994a; De Feo *et al.*, 2009; Klomp *et al.*, 2003; Lee *et al.*, 2002; Peña *et al.*, 2000; Puig *et al.*, 2002). A conserved Gly-$X_3$-Gly motif within TMD3 is essential for the Ctr1 proteins to appropriately oligomerize (Aller *et al.*, 2004). Studies have shown that despite the cytosolic carboxyl-terminal domain (CTD) of *S. cerevisiae* Ctr1 protein being dispensable for Cu transport activity, it is important for the post-transcriptional regulation of the protein in response to increasing exogenous Cu concentrations (Liu *et al.*, 2007; Ooi *et al.*, 1996; Wu *et al.*, 2009). Ctr1 has been proposed to undergo Cu-induced degradation at the plasma membrane, as well as endocytosis and degradation at the vacuole (Liu *et al.*, 2007; Ooi *et al.*, 1996). More recently, the Ctr1 CTD has been implicated in the regulation of the Cu transport activity of the protein on the cell surface (Wu *et al.*, 2009). In the presence of Cu, it has been suggested that some residues within the Ctr1 CTD trigger a conformational change which inactivates Ctr1 Cu transport activity in order to protect cells against toxic Cu levels (Wu *et al.*, 2009). Consequently, *S. cerevisiae* cells expressing a truncated Ctr1 protein lacking amino acid residues within its CTD are more sensitive to high Cu concentrations (Wu *et al.*, 2009).

We previously identified the *Schizosaccharomyces pombe* Ctr4 and Ctr5 proteins, structurally related to the Ctr1 family, as a high-affinity Cu transport complex (Labbé *et al.*, 1999; Zhou & Thiele, 2001). The heterologous expression of Ctr4 in the *S. cerevisiae ctr1*Δ*ctr3*Δ mutants fails to rescue Cu transport activity, and the Ctr4 protein is trapped within the secretory pathway of the budding yeast (Zhou & Thiele, 2001). A genetic screen using the *S. cerevisiae ctr1*Δ*ctr3*Δ strain allowed the identification of *Sch. pombe* Ctr5, which rescues both the localization of Ctr4 to the plasma membrane and its Cu-transporting activity in *S. cerevisiae* (Zhou & Thiele, 2001). Subsequent studies in *Sch. pombe* have revealed that the expression of the $ctr4^+$ and $ctr5^+$ genes is induced in response to Cu deficiency by the Cuf1 Cu-sensing transcription factor (Beaudoin & Labbé, 2001; Zhou & Thiele, 2001). Furthermore, unlike the *S. cerevisiae* Ctr1 and Ctr3 proteins, the Ctr4 and Ctr5 proteins physically interact with each other to form a heteromeric complex, and they are interdependent for secretion to the plasma membrane and Cu transport activity (Beaudoin *et al.*, 2006; Zhou & Thiele, 2001). Similar to other high-affinity Cu transport proteins of the Ctr1 family, Ctr4 and Ctr5 display amino-terminal domains (NTDs) rich in Mets motifs. Although they are not essential for the Cu transport function, the Mets motifs within the NTDs of both *Sch. pombe* proteins enhance Cu uptake efficiency in an independent yet redundant manner (Beaudoin *et al.*, 2006). Furthermore, the recent results of a bimolecular fluorescence complementation assay strongly suggest that the assembly of a functional high-affinity Cu uptake system on the cell surface of *Sch. pombe* requires the combination of two Ctr4 molecules with one Ctr5 molecule (Ioannoni *et al.*, 2010). Moreover, these studies have demonstrated that the Ctr4–Ctr5 complex is post-transcriptionally regulated by Cu (Ioannoni *et al.*, 2010), with both proteins being internalized in response to high Cu concentrations and recycled back to the cell surface when Cu availability is diminished.

The *Sch. pombe* Ctr4 and Ctr5 proteins contain all the domains known to be required for the Cu-transporting activity of the Ctr1 family of Cu transporters, including three TMDs, the extracellular Mets motifs, the conserved methionine residues within the NTD, the Met-$X_3$-Met motif within TMD2, and the TMD3 Gly-$X_3$-Gly motif (Fig. 1). Notwithstanding, Ctr4 and Ctr5 are unable to function independently in Cu acquisition. In this study, we used the yeasts *S. cerevisiae* and *Sch. pombe* to study the contri-

bution of Ctr4 and Ctr5 to the Cu transport activity of the heteromeric complex. The mutagenesis of the essential motifs for transport suggests that Ctr5 exerts an accessory function in the Cu transporter complex. Furthermore, the functional dissection of the Ctr4 and Ctr5 domains using Ctr4/Ctr5 chimeric proteins shows that the Ctr4 CTD exerts an inhibitory effect on trafficking the Ctr4 protein to the cell surface, and that this is rescued by the Ctr5 CTD.

## METHODS

**Yeast strains and growth conditions.** The MPY17 strain, lacking both the Ctr1 and Ctr3 high-affinity Cu transporters (Peña *et al.*, 1998), was used for the functional complementation assays and subcellular localization studies in *S. cerevisiae*. The MPY17 strain was cotransformed with the centromeric p413GPD and p416GPD vectors (Mumberg *et al.*, 1995), either empty or expressing wild-type or modified alleles of the *CTR1*, *CTR4* and *CTR5* genes. The transformed *S. cerevisiae* cells were maintained in a synthetic complete (SC) medium lacking uracil and histidine (SC-ura-his) (Kaiser *et al.*, 1994). The *ctr4*Δ*ctr5*Δ strain, isogenic to FY435 (*h*$^+$ *his7-366 leu1-32 ura4-*Δ*18 ade6-M210*) (Bezanilla *et al.*, 1997) and to *ctr4*::*ura4*$^+$ *ctr5*::*Kan*$^R$, was used for the functional complementation assays and subcellular localization studies in *Sch. pombe*. The *ctr4*Δ*ctr5*Δ strain was cotransformed with the pBPAde6 (*ade6*$^+$) and pJK148 (*leu1*$^+$) vectors (Beaudoin *et al.*, 2006; Keeney & Boeke, 1994), which either were empty or expressed wild-type or modified alleles of the *ctr4*$^+$ and *ctr5*$^+$ genes. The transformed *Sch. pombe* cells were maintained in a selective Edinburgh minimal medium with all the auxotrophic requirements, except adenine and leucine (EMM-ade-leu) (Alfa *et al.*, 1993).

**Plasmids.** *Escherichia coli* was used for the construction of recombinant plasmids. Transformants were grown at 37 °C in LBA medium with 50 μg ampicillin ml$^{-1}$ (Sambrook *et al.*, 1989). For expression in *S. cerevisiae*, the coding sequences of the *ctr4*$^+$ and *ctr5*$^+$ genes were PCR-amplified and cloned into the polylinker of the p413GPD and p416GPD vectors (Mumberg *et al.*, 1995), respectively.

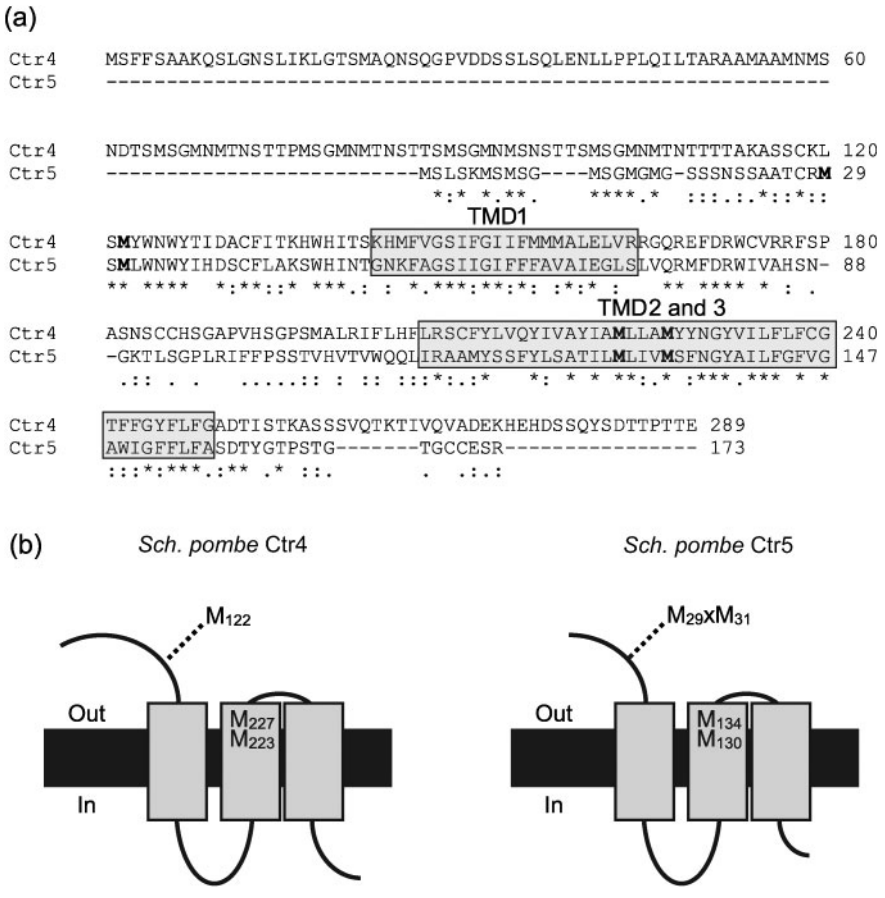


**Fig. 1.** Notable features of *Sch. pombe* Ctr4 and Ctr5 proteins. (a) Sequence alignment and primary structural features of Ctr4 and Ctr5 proteins. The alignment was performed with CLUSTAL W2 software. Asterisks indicate the presence of an identical amino acid in both proteins, whereas dots indicate similarity between amino acids. (b) Schematic representation of the putative topology of Ctr4 and Ctr5 proteins. According to the topology displayed by the human and *S. cerevisiae* Ctr1 proteins, we propose that the amino-termini of Ctr4 and Ctr5 proteins would localize to the extracellular medium and that their carboxyl-termini would face the cytosol. Grey-shaded boxes represent the predicted locations of membrane-spanning domains (TMDs). The methionine residues located in conserved positions, which are essential for the Cu transport activity in human and *S. cerevisiae* Ctr1 proteins, are shown in bold type. The amino acid sequence number relating to the first amino acid of the protein is indicated.

For GFP-tagging at the carboxyl-terminus, the coding sequences of the wild-type, mutant and chimeric *ctr4*$^+$ and *ctr5*$^+$ genes were amplified without their stop codon, and cloned into the p413GPD and p416GPD vectors, respectively, between the *Bam*HI and *Eco*RI sites. The GFP coding sequence, including a stop codon, was PCR-amplified and cloned in-frame between the *Eco*RI and *Sal*I sites. For expression in *Sch. pombe*, the pSP1-CTR4-GFP and pSP2-CTR5-GFP plasmids (Zhou & Thiele, 2001) were used as templates for the PCR amplifications. The coding sequences of the wild-type, mutant and chimeric *ctr4*$^+$ and *ctr5*$^+$ genes (with or without GFP) were cloned into the polylinker of the pBPAde6 and pJK148 vectors, respectively. In all cases, the overlap extension method (Ho *et al.*, 1989) was followed for the mutagenesis of specific methionine residues and the construction of the Ctr4/Ctr5 chimeric proteins.

**Functional complementation assays.** Transformed *S. cerevisiae* MPY17 cells were grown to exponential phase ($OD_{600}$ ~1.0) in liquid SC-ura-his medium, and plated as drops ($OD_{600}$ 0.1, and serial 10-fold dilutions) on solid SC-ura-his medium with or without 50 μM bathophenanthroline disulfonic acid (BPS), on rich medium with glucose (YPD) (Kaiser *et al.*, 1994) with or without 100 μM BPS, and on rich medium with ethanol/glycerol (YPEG) (Puig *et al.*, 2002) with or without either 100 μM $CuSO_4$ or 100 μM bathocuproine disulfonic acid (BCS). The transformed *Sch. pombe ctr4*Δ*ctr5*Δ cells were grown to the exponential phase ($OD_{600}$ ~1.0) in liquid EMM-ade-leu medium with 100 μM BCS, and plated as drops on a solid yeast extract plus supplements (YES) medium (Alfa *et al.*, 1993), and a respiratory carbon source YES/glycerol/ethanol (YES-EG) medium in which glucose was replaced with 3 % (v/v) glycerol and 2 % ethanol. Plates were incubated at 30 °C for 3–7 days and photographed.

**Fluorescence microscopy.** *S. cerevisiae* cells were grown to exponential phase in liquid SC-ura-his medium, and visualized with a confocal fluorescence microscope as described previously (Puig *et al.*, 2002). *Sch. pombe* cells were grown to exponential phase in liquid EMM-ade-leu medium with 100 μM BCS, and visualized with a Nikon Eclipse E800 epifluorescent microscope equipped with a Hamamatsu ORCA-ER (charge-coupled device; CCD) camera as described previously (Beaudoin *et al.*, 2006).

## RESULTS

### Integrity of the Ctr4 Met-$X_3$-Met motif is essential for the Cu transport activity of the Ctr4–Ctr5 complex in *S. cerevisiae*

A conserved Met-$X_3$-Met motif within TMD2 is indispensable for the Cu transport function of all the Ctr1 family of high-affinity Cu transporters studied, which includes the *S. cerevisiae* Ctr1 and Ctr3 proteins, as well as the vacuolar Ctr2 protein and human Ctr1 (Puig *et al.*, 2002; Rees *et al.*, 2004). Therefore, to ascertain the relative contributions of Ctr4 and Ctr5 to the Cu transport activity of the *Sch. pombe* Ctr4–Ctr5 complex, we performed the following assay. First, we substituted the Met$^{223}$ and Met$^{227}$ in Ctr4 and the Met$^{130}$ and Met$^{134}$ in Ctr5 with alanine residues by generating the *CTR4-M223/227A* and *CTR5-M130/134A* mutant alleles, respectively. Then, we cloned both alleles into vectors designed for constitutive expression in *S. cerevisiae* (Mumberg *et al.*, 1995). With this strategy, we assayed protein functionality independently of the Cu regulation of the gene expression by the endogenous promoter. We expressed both mutant alleles, simultaneously or in combination with their wild-type counterpart, in the *S. cerevisiae ctr1*Δ*ctr3*Δ strain. As previously reported (Puig *et al.*, 2002), the *ctr1*Δ*ctr3*Δ mutant grew neither in medium with ethanol and glycerol (YPEG) as the sole carbon source nor in medium with low Fe availability, as achieved by the addition of the $Fe^{2+}$-specific chelator BPS (Fig. 2). Yeast growth was rescued if the Fe chelator was removed from YPD medium, or if excess Cu was added to YPEG medium (Fig. 2) (Puig *et al.*, 2002). Furthermore, whereas the independent expression of *CTR4* and *CTR5* did not restore growth of the *ctr1*Δ*ctr3*Δ mutant in the presence of BPS and YPEG, their co-expression achieved growth rates similar to those obtained with *S. cerevisiae CTR1* expression (Fig. 2) (Zhou & Thiele, 2001). Interestingly, the co-expression of wild-type *CTR4* with the *CTR5-M130/134A* mutant allele in strain *ctr1*Δ*ctr3*Δ allowed growth in the presence of BPS and YPEG (Fig. 2a). This result indicates either that the Ctr5 Met-$X_3$-Met motif is not required for the Cu transport activity of the Ctr4–Ctr5 complex, or that the Met-$X_3$-Met motif within only one of the two proteins is required for function. The co-expression of the *CTR4-M223/227A* mutant allele with wild-type *CTR5* indicates that the Met-$X_3$-Met motif within Ctr4 TMD2 is absolutely necessary for *ctr1*Δ*ctr3*Δ growth in YPEG, and growth was strongly diminished in medium containing BPS to levels similar to those achieved by the expression of *CTR4* alone (Fig. 2a). Finally, the co-expression of both mutant alleles, *CTR4-M223/227A* and *CTR5-M130/134A*, completely abrogated growth in the presence of BPS and YPEG. Taken together, these results indicate that the Met-$X_3$-Met motif in Ctr5 is dispensable for the functionality of the Ctr4–Ctr5 complex, whereas the Met-$X_3$-Met motif in Ctr4 is essential for Cu transport.

To further dissect the relative contributions of Met$^{223}$ and Met$^{227}$ in the Ctr4 Met-$X_3$-Met motif to the Cu transport activity of the Ctr4–Ctr5 complex, we separately substituted the Ctr4 Met$^{223}$ and Met$^{227}$ residues with alanine. The constitutive co-expression of either the *CTR4-M223A* or the *CTR4-M227A* mutant allele in combination with wild-type *CTR5* in the *ctr1*Δ*ctr3*Δ mutant suggests that the mutagenesis of Ctr4 Met$^{227}$ does not affect growth on BPS and YPEG, whereas the substitution of Ctr4 Met$^{223}$ strongly limited yeast growth in both BPS and YPEG media. The effect of Met$^{227}$ mutagenesis on the Ctr4–Ctr5 Cu transport function was only observed when cells were grown on YPEG containing the $Cu^+$-specific chelator BCS, or when combined with the Met$^{223}$ mutation (Fig. 2b). These results indicate that both methionine residues in the Ctr4 Met$^{223}$-$X_3$-Met$^{227}$ motif contribute to the Cu transport activity of the heteromeric complex, with a predominant role for the Met$^{223}$ residue.

The lack of functionality of the Ctr4–Ctr5 complex expressing a *CTR4* allele mutated at its Met-$X_3$-Met motif could either be due to a defect in the Cu transport activity on the cell surface or be the result of a defect in the delivery

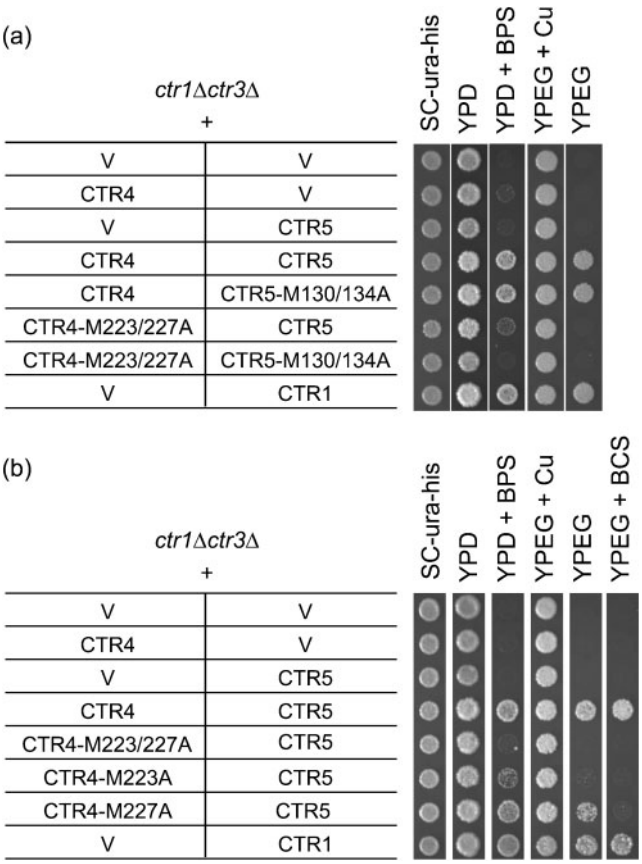


**Fig. 2.** The Ctr5 Met-$X_3$-Met motif is not required for the function of the Ctr4–Ctr5 complex in *S. cerevisiae*. (a) Analysis of the role of the Met-$X_3$-Met motif in Ctr4 and Ctr5 function. The *S. cerevisiae ctr1*Δ*ctr3*Δ deletion mutant was cotransformed with combinations of vector (V), *CTR4* (wild-type, left column), *CTR4-M223/227A*, *CTR5* (wild-type, right column), *CTR5-M130/134A* and *CTR1*, as indicated. Cells were spotted onto SC-ura-his, YPD, YPD with 100 μM BPS, YPEG and YPEG with 100 μM $CuSO_4$, grown for 3 days at 30 °C, and photographed. (b) Dissection of the relative contribution of the $Met^{223}$ and $Met^{227}$ residues of Ctr4 to its Cu transport function. The *ctr1*Δ*ctr3*Δ deletion mutant was cotransformed with the indicated combinations of vector (V), *CTR4* (wild-type, left column), *CTR5* (wild-type, right column), *CTR4-M223/227A*, *CTR4-M223A*, *CTR4-M227A* and *CTR1*. In addition to the media used in (a), cells were also spotted onto YPEG with 100 μM BCS.

of the heteromeric complex to the plasma membrane. To distinguish between these two possibilities, we determined the subcellular localization of the wild-type and the mutated GFP-tagged Ctr4 and Ctr5 proteins in the *S. cerevisiae ctr1*Δ*ctr3*Δ strain. As previously described (Zhou & Thiele, 2001), Ctr4 and Ctr5 were intracellularly trapped in a perinuclear structure that could correspond to the early secretory pathway when expressed without their complex counterpart (Fig. 3a, b). Furthermore, a predominant cell surface localization was observed for both wild-type proteins when simultaneously co-expressed in budding yeast (Fig. 3c, d) (Zhou & Thiele, 2001). Interestingly, the mutagenesis of the Ctr5 Met-$X_3$-Met motif did not significantly affect the localization of Ctr4 and Ctr5 to the plasma membrane (Fig. 3e, f). Although the expression of the Ctr4-M223/227A protein slightly increased the intracellular localization of Ctr4 and Ctr5, consistent with the cell growth phenotypes, a considerable amount of both proteins still reached the cell surface (Fig. 3g, h). Finally, the co-expression of both Ctr4-M223/227A and Ctr5-M130/134A did not alter the localization of these proteins to the plasma membrane, with a significant proportion of the heterocomplex found at the cell surface (Fig. 3i, j). Taken together, these results demonstrate that the mutagenesis of the Met-$X_3$-Met motif in Ctr4 and Ctr5 does not abrogate their localization at the surface of the cell. Furthermore, these data strongly suggest that the growth defect displayed by the cells expressing the *CTR4-*$Met_{223}$*-*$X_3$*-*$Met_{227}$ mutant alleles under low Fe and in the presence of non-fermentable carbon sources is due to a lack of Cu transport function rather than to a defect in trafficking of the heteroprotein complex to the plasma membrane.

## Expression of a Ctr4/Ctr5 chimeric protein confers Cu transport activity in *S. cerevisiae*

The results obtained from the mutagenesis of the Met-$X_3$-Met motifs located in TMD2 suggest that Ctr4 plays a major role in the mechanism that transports Cu across the plasma membrane lipid bilayer, whereas the Ctr5 Met-$X_3$-Met motif does not directly participate in Cu transport activity. Previous data have demonstrated that the extracellular Mets motifs of both proteins contribute to efficient Cu assimilation by the Ctr4–Ctr5 heteromeric complex (Beaudoin *et al.*, 2006). In order to investigate the relative contributions of the different Ctr4 and Ctr5 protein domains to the Cu transport activity of the heteromeric complex, we constructed chimeric proteins between Ctr4 and Ctr5, and tested the chimeras for Cu transport activity. A crucial point in this approach was the choice of the most appropriate position to perform the sequence swap between Ctr4 and Ctr5 in order to minimize any potential alteration of the overall transporter structure. We swapped the protein sequences at two conserved positions which are essential in the *S. cerevisiae* Ctr1 protein (Puig *et al.*, 2002). The first swap position localizes to the methionine residue located 20 aa before TMD1, which is $Met^{122}$ in Ctr4 and $Met^{31}$ in Ctr5 (Fig. 1). The second swap point was located in the last methionine residue of the Met-$X_3$-Met motif of both proteins, which is $Met^{227}$ for Ctr4 and $Met^{134}$ for Ctr5 (Fig. 1). Therefore, we divided each protein into three distinct regions: the extracellular NTD [Ctr4(1–122) and Ctr5(1–31)], which contains the Mets motifs; the central region [Ctr4(122–227) and Ctr5(31–134)], which extends over TMD1, the cytosolic loop and TMD2, including the Met-$X_3$-Met motif; and the CTD [Ctr4(227–289) and Ctr5(134–173)], which covers TMD3, including the Gly-$X_3$-Gly motif and the intracellular carboxyl-terminus (Fig.

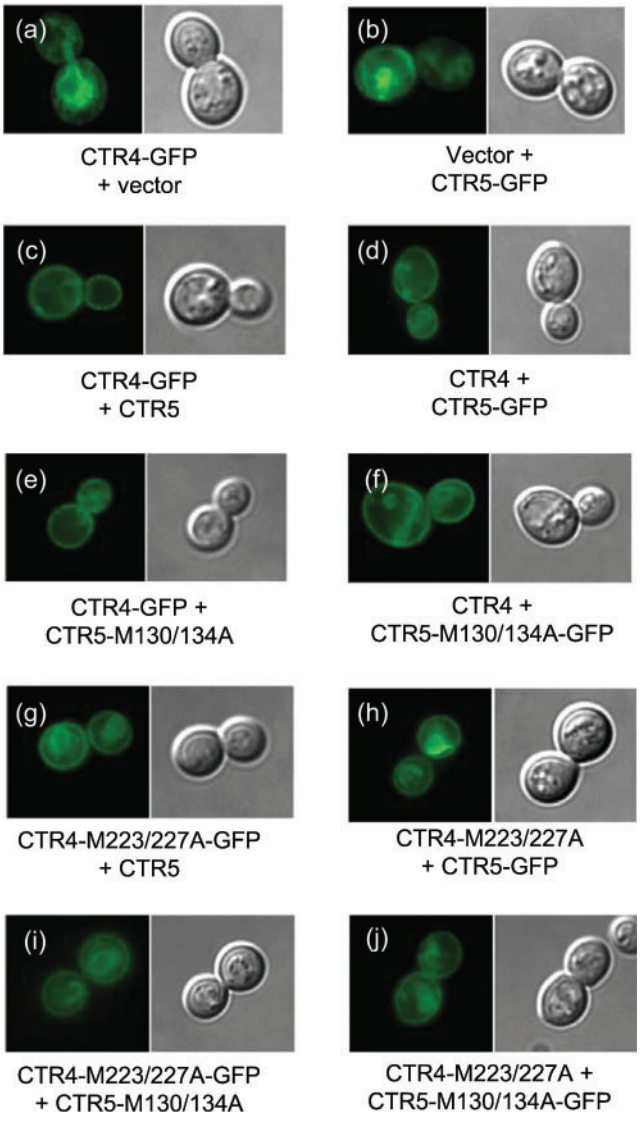


**Fig. 3.** Subcellular localization of the Ctr4 and Ctr5 mutant proteins in *S. cerevisiae*. The *ctr1*Δ*ctr3*Δ deletion mutant was cotransformed with combinations of vector, *CTR4*, *CTR4-GFP*, *CTR4-M223/227A*, *CTR4-M223/227A-GFP*, *CTR5*, *CTR5-GFP*, *CTR5-M130/134A* and *CTR5-M130/134A-GFP*, as indicated in the different panels. Cells were grown to exponential phase, and then visualized for GFP by fluorescence microscopy. Representative images of four separate experiments are shown. Cell morphology was examined through Nomarski optics.

1). Thereby, we obtained six different Ctr4/Ctr5 chimeric proteins, three with a Ctr5 central region, denoted Ctr455, Ctr554 and Ctr454; plus three additional chimeras with a Ctr4 central region, denoted Ctr544, Ctr445 and Ctr545 (Fig. 4a, b). To assess their function in Cu transport, we used the growth assay in the *S. cerevisiae ctr1*Δ*ctr3*Δ strain, as described above. As shown in Fig. 4(c), none of the chimeras with a Ctr5 central region was able to rescue the growth defect of the *ctr1*Δ*ctr3*Δ strain in BPS or YPEG medium. This result is consistent with the essential function of the central domain of the Ctr4 protein, which contains the key Met-$X_3$-Met motif that is not present in these chimeric proteins (Fig. 4a). An identical result was obtained for the Ctr544 chimera (Fig. 4d), probably due to the lack of a Ctr5 counterpart. One interesting finding for the chimeric proteins containing the central region of Ctr4 and the Ctr5 CTD, i.e. Ctr445 and Ctr545, was that they fully complemented the growth defect of the *ctr1*Δ*ctr3*Δ cells in BPS or YPEG medium, and did not require any Ctr4 or Ctr5 counterpart for the function (Fig. 4d). Taken together, these data demonstrate that the expression of a Ctr4/Ctr5 chimeric protein suffices to complement the growth defects of the *S. cerevisiae ctr1*Δ*ctr3*Δ strain, perhaps because it comprises the essential functional properties separately contained in the individual Ctr4 and Ctr5 polypeptides.

To ascertain whether the distinct functionality obtained for the expression of the different Ctr4/Ctr5 chimeric proteins in the *S. cerevisiae ctr1*Δ*ctr3*Δ strain resulted from either a Cu transport malfunction or a defect in the delivery of the protein to the plasma membrane, the chimeric proteins were epitope-tagged with GFP at the carboxyl-terminus and their subcellular localization was ascertained by fluorescence microscopy. As a control, wild-type Ctr4-GFP and Ctr5-GFP were separately or simultaneously expressed (Fig. 5a–d). All of the Ctr4/Ctr5 chimeric proteins that did not complement the *ctr1*Δ*ctr3*Δ strain (Ctr455, Ctr554, Ctr454 and Ctr544) displayed an intracellular localization pattern (Fig. 5e, f, h and j), and the Ctr4/Ctr5 chimeras complementing the *ctr1*Δ*ctr3*Δ strain (Ctr445 and Ctr545) localized to the cell surface, similar to the result observed for co-expression of wild-type Ctr4-GFP and Ctr5-GFP (Fig. 5c, d, g, i). These results show that the functionality of the Ctr4/Ctr5 chimera directly correlates with its subcellular localization in *S. cerevisiae*. Furthermore, these data strongly suggest that the information for the correct delivery of the Ctr4–Ctr5 complex to the *S. cerevisiae* plasma membrane for Cu transport function is contained in both the Ctr4 central domain and the Ctr5 CTD, which are present in the Ctr4–Ctr5 heteromeric complex, and also in chimeric proteins Ctr445 and Ctr545.

The *S. cerevisiae* Ctr1 protein possesses two motifs which are indispensable for Cu acquisition and which contain key methionine residues, the Met-$X_3$-Met motif, and the extracellular $Met^{127}$ located at approximately 20 aa before TMD1 (Puig *et al.*, 2002). As shown above, the mutagenesis of the Met-$X_3$-Met motif in Ctr4 abolishes the Cu transport function of the Ctr4–Ctr5 complex in *S. cerevisiae*. However, as previously reported (Beaudoin *et al.*, 2006), the mutagenesis of the extracellular Ctr4 $Met^{122}$ residue, which is the equivalent of $Met^{127}$ in Ctr1, does not eliminate the Cu transport activity of the Ctr4–Ctr5 complex, perhaps due to the redundant function of the corresponding extracellular methionine residues in Ctr5 ($Met^{29}$-X-$Met^{31}$). Here, we show that a single protein,

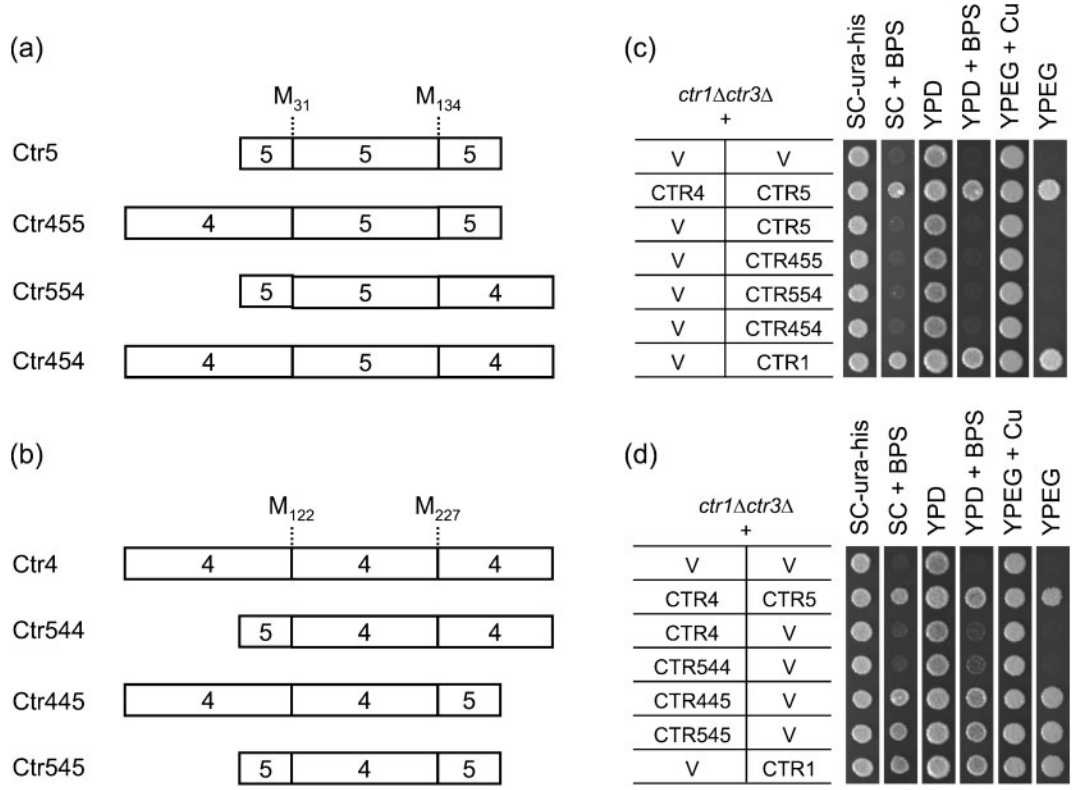


**Fig. 4.** Functionality of the Ctr4 and Ctr5 protein chimeras in *S. cerevisiae*. (a, b) Schematic representation of Ctr4/Ctr5 chimeras. The Ctr4 and Ctr5 proteins were divided into three regions at the conserved Ctr4 $Met^{122}$ and $Met^{227}$ residues, and at the Ctr5 $Met^{31}$ and $Met^{134}$ residues. All the possible combinations between the Ctr4 and Ctr5 protein regions were constructed, including the Ctr4-based chimeras CTR544, CTR445 and CTR545 (a), and the Ctr5-based chimeras CTR455, CTR554 and CTR454 (b). (c, d) The *S. cerevisiae ctr1*Δ*ctr3*Δ deletion mutant was cotransformed with combinations of vector (V), *CTR4* (wild-type, left column), *CTR544*, *CTR445*, *CTR545*, *CTR5* (wild-type, right column), *CTR455*, *CTR554*, *CTR454* and *CTR1*, as indicated. In addition to the media assayed in Fig. 2(a), cells were spotted onto SC with 50 μM BPS. Cells were grown for 3 days at 30 °C and photographed.

Ctr445 (and also Ctr545), is able to complement the defects of the *S. cerevisiae ctr1*Δ*ctr3*Δ strain (Fig. 4d). If these Ctr4/Ctr5 chimeric proteins assembled as homotrimers, as previously reported for other Ctr1 family members (Aller & Unger, 2006; Lee *et al.*, 2002; Peña *et al.*, 2000), we would expect the mutagenesis of the $Met^{122}$ residue within the NTD of Ctr445 to completely eliminate its Cu transport activity. Indeed, the *CTR445-M122A* mutant allele does not allow the *ctr1*Δ*ctr3*Δ cells to grow in YPEG or in medium with low Fe availability (Supplementary Fig. S1). This result suggests that the $Met^{122}$ residue is essential for the Cu transport activity of Ctr445 in *S. cerevisiae*.

## Expression in *Sch. pombe* corroborates the results obtained in *S. cerevisiae*

To ascertain whether the results obtained via heterologous expression in *S. cerevisiae* cells were also applicable to *Sch. pombe*, we expressed the distinct $ctr4^+$ and $ctr5^+$ alleles in a *ctr4*Δ*ctr5*Δ fission yeast mutant. As previously reported (Zhou & Thiele, 2001), the simultaneous expression of $ctr4^+$ and $ctr5^+$ is required for *Sch. pombe* growth in media containing non-fermentable carbon sources, such as glycerol and ethanol (YES-EG) (Fig. 6). Growth of the *ctr4*Δ*ctr5*Δ strain in YES-EG medium was fully rescued by the addition of excess Cu (Fig. 6a). Similar to our results in budding yeast, the co-expression of the *CTR4-M223/227A* mutant allele with wild-type $ctr5^+$ did not support *Sch. pombe ctr4*Δ*ctr5*Δ growth in YES-EG (Fig. 6a). Localization of a Ctr4-M223/227A-GFP fusion protein in the *ctr4*Δ*ctr5*Δ fission yeast mutant co-expressing wild-type $ctr5^+$ indicated that it was mostly trapped intracellularly (Fig. 7d). Furthermore, the mutagenesis of the $Met^{130}$ and $Met^{134}$ residues within Ctr5 had no effect on *Sch. pombe* growth in YES-EG (Fig. 6a). Taken together, these results demonstrate that the Met-$X_3$-Met motif in protein Ctr4 is essential for the functionality of the Ctr4–Ctr5 complex on the cell surface of *Sch. pombe*, whereas the Met-$X_3$-Met motif in Ctr5 is dispensable for function.

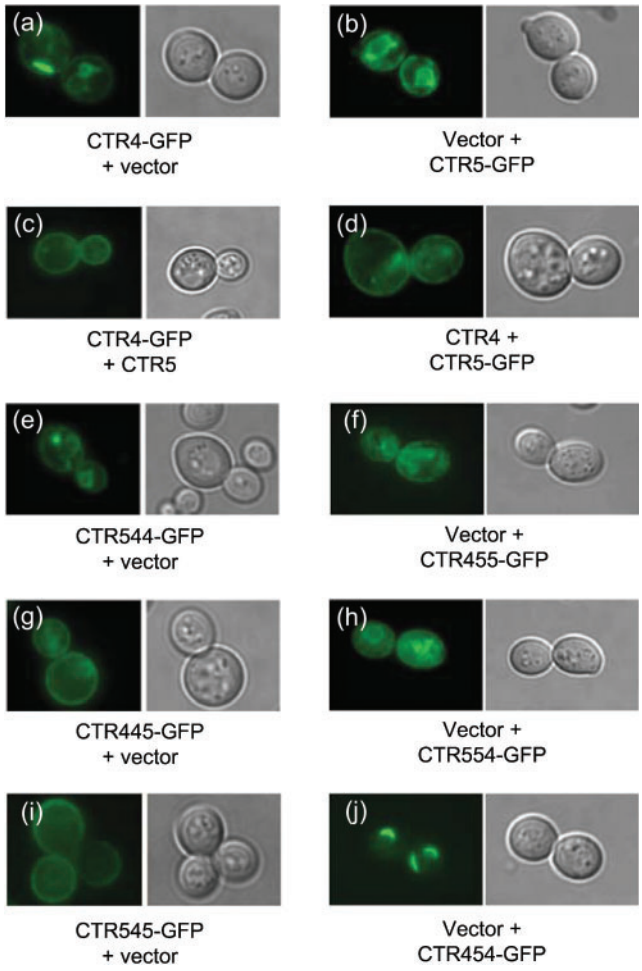


**Fig. 5.** Subcellular localization of the Ctr4 and Ctr5 protein chimeras in *S. cerevisiae*. The *S. cerevisiae ctr1*Δ*ctr3*Δ deletion mutant was cotransformed with combinations of vector, *CTR4*, *CTR4-GFP*, *CTR544-GFP*, *CTR445-GFP*, *CTR5*, *CTR5-GFP*, *CTR545-GFP*, *CTR455-GFP*, *CTR554-GFP* and *CTR454-GFP*, as indicated in the different panels. Representative images of four separate experiments are shown. Cells were grown and analysed as in Fig. 3.

To ascertain whether the expression of a single Ctr4/Ctr5 chimeric protein is also sufficient to promote Cu transport activity in *Sch. pombe*, we expressed the *CTR445* chimera, fused to GFP, in a *ctr4*Δ*ctr5*Δ strain. As Fig. 6(b) illustrates, *CTR445* complemented the growth defects of the *Sch. pombe* strain in YES-EG. Furthermore, protein localization studies demonstrated that Ctr445-GFP reaches the plasma membrane in *ctr4*Δ*ctr5*Δ *Sch. pombe* cells without requiring any accessory counterpart (Fig. 7e). Taken together, these data strongly suggest that the CTD of Ctr4 inhibits the delivery of the protein to the cell surface in *Sch. pombe*. The substitution of the Ctr4 CTD with the Ctr5 CTD, or the simultaneous expression of $ctr5^{+}$, leads to the localization of Ctr4 to the cell surface of *Sch. pombe* cells, where it mediates high-affinity Cu transport.

## DISCUSSION

The Ctr1 family of Cu transporters mediates high-affinity Cu uptake in eukaryotic organisms. Recent structural studies have demonstrated that the human Ctr1 complex assembles as a symmetrical homotrimer with a cone-shaped pore in the centre (Aller & Unger, 2006; De Feo *et al.*, 2009). The conserved TMD2 Met-$X_3$-Met motif, which is essential for function, is located at the narrowest end (~8 Å) of the Ctr1 pore toward the extracellular face of the lipid bilayer (De Feo *et al.*, 2009). The close proximity between the Met-$X_3$-Met motifs from different Ctr1 monomers leads to the formation of disulfide bonds in cross-linking experiments when a methionine residue at the Met-$X_3$-Met motif is substituted by a cysteine (De Feo *et al.*, 2009). These results, and the *in vivo* functionality of the *S. cerevisiae* Ctr1 $Met^{256}$-$X_3$-$Cys^{260}$ mutant (Puig *et al.*, 2002), suggest that $Cu^{+}$ simultaneously coordinates to the methionine thioether residues from different Ctr1 monomers during metal ion passage through the pore. The results of genetic studies with *S. cerevisiae* Ctr1 are also consistent with the cooperation between different monomers of the assembled complex during Cu transport. Whereas *S. cerevisiae* cells expressing a Ctr1 protein with a substitution of either the extracellular $Met^{127}$ residue or the $Met^{260}$ in the $Met^{256}$-$X_3$-$Met^{260}$ motif for alanine are unable to transport Cu, yeast cells co-expressing both mutants display significant Cu uptake capacity (Puig *et al.*, 2002). The *Sch. pombe* Cu transporter, which is composed of two distinct Ctr1-like proteins (Ctr4 and Ctr5), is an excellent system to dissect the contribution of each monomer to the Cu transport complex. Although both proteins contain all known critical motifs described for the Ctr1 family of high-affinity Cu transporters, they are unable to function independently in Cu acquisition (Zhou & Thiele, 2001). Recent bimolecular fluorescence assays in *Sch. pombe* point to the assembly of two Ctr4 molecules in combination with one Ctr5 molecule (Ioannoni *et al.*, 2010). According to this ratio, the mutagenesis of the Ctr4 Met-$X_3$-Met motif would leave a single motif in the complex, whereas the substitution of the Ctr5 Met-$X_3$-Met motif would leave two functional motifs. Given that the mutagenesis of the Ctr4 Met-$X_3$-Met motif abolishes Cu transport, whereas Ctr5 Met-$X_3$-Met mutagenesis does not, we propose that cooperation between at least two functional Met-$X_3$-Met motifs is necessary for Cu transport across the Ctr4–Ctr5 complex at the plasma membrane.

To further dissect the contribution of the distinct Ctr4 and Ctr5 protein domains to the assembly of an active heteromeric complex on the cell surface, we constructed six different Ctr4/Ctr5 protein chimeras and assayed for Cu transport functionality. Only two chimeras, Ctr445 and Ctr545, were able to rescue the respiratory defect of a yeast strain defective in high-affinity Cu uptake without a Ctr4 or Ctr5 counterpart. These data suggest that both Ctr4 and Ctr5 NTDs are compatible with a functional chimera, and this result is consistent with the redundant and independent functions of the Ctr4 and Ctr5 amino-terminal Mets

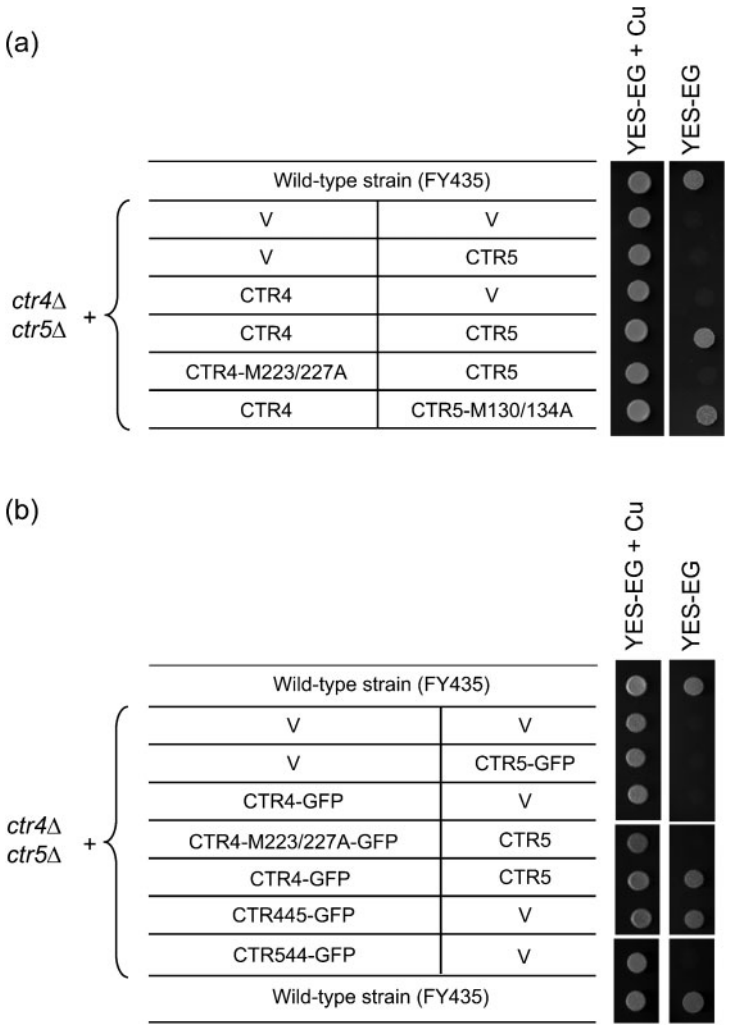


**Fig. 6.** Functionality of the Ctr4 and Ctr5 protein mutants and chimeras in *Sch. pombe*. (a) The Ctr5 Met-$X_3$-Met motif is not required for the function of the Ctr4–Ctr5 complex in *Sch. pombe*. The *Sch. pombe ctr4*Δ*ctr5*Δ deletion strain was cotransformed with combinations of vector (V), *CTR4* (left column), *CTR5* (right column), *CTR4-M223/227A* and *CTR5-M130/134A*, as indicated. Wild-type (FY435) and the transformed *ctr4*Δ*ctr5*Δ *Sch. pombe* isogenic cells were spotted onto YES agar media containing glycerol/ethanol without (YES-EG) or with 15 μM $CuSO_4$ (YES-EG + Cu). (b) Expression of a Ctr445 chimeric protein complements the *ctr4*Δ*ctr5*Δ respiratory defect. The *Sch. pombe ctr4*Δ*ctr5*Δ deletion strain was cotransformed with combinations of vector (V), *CTR4*, *CTR4-GFP*, *CTR4-M223/227-GFP*, *CTR5*, *CTR5-GFP*, *CTR5*, *CTR445-GFP* and *CTR544-GFP*, as indicated. *Sch. pombe* cells were grown at 30 °C and photographed.

motifs in efficient Cu transport (Beaudoin *et al.*, 2006). Previous results have also revealed that the simultaneous mutagenesis of both extracellular methionine residues within Ctr4 and Ctr5 NTDs is necessary to abolish Cu transport function in *Sch. pombe*, whereas the substitution of one does not abrogate Cu transport (Beaudoin *et al.*, 2006). Consistent with these results and with previous data on the lack of functionality of the yeast Ctr1-$Met^{127}$ mutant (Puig *et al.*, 2002), the single substitution of $Met^{122}$ for alanine eliminates the Cu transport function of the Ctr445 chimera, but not that of the Ctr4–Ctr5 complex. Taken together, these studies suggest that a single methionine-rich NTD is necessary and sufficient for Ctr4–Ctr5 Cu-mediated transport.

The functionality of the Ctr445 and Ctr545 chimeras highlights the fact that only the Ctr4 central domain and the Ctr5 CTD are appropriate for an independent functional chimera. The central domain comprises the TMD1, the cytosolic loop, and the TMD2 containing the Met-$X_3$-Met motif. The lack of cell surface localization for Ctr5, expressed without a Ctr4 counterpart, as compared with the peripheral localization of the Ctr545 chimera, suggests that the amino acid composition of the Ctr5 central domain abrogates the delivery to the plasma membrane, probably as a result of defects in the assembly of the trimer complex. Regarding Ctr4–Ctr5 CTDs, Ctr4 is trapped during secretion when expressed without a Ctr5 counterpart, whereas the Ctr445 chimera localizes to the plasma membrane and functions in Cu transport. These results suggest that the Ctr4 CTD, which is composed of TMD3 and the cytosolic amino-terminal tail, exerts a negative effect on the delivery of the protein to the cell surface that is antagonized by the Ctr5 CTD.

Yeast cells expressing the Ctr445 chimeric protein show a significant growth defect, as compared with cells expressing the wild-type Ctr4–Ctr5 heteromeric complex, when the Cu availability of a medium containing non-fermentable carbon sources is decreased by the addition of the Cu chelator BCS (data not shown). This observation is consistent with a slight defect in Ctr445 functionality as compared with the Ctr4–Ctr5 native complex, and suggests that the Ctr4/Ctr5 chimeric proteins do not retain all the biological properties of the wild-type Ctr4–Ctr5 complex. Although most of the structural and functional character-

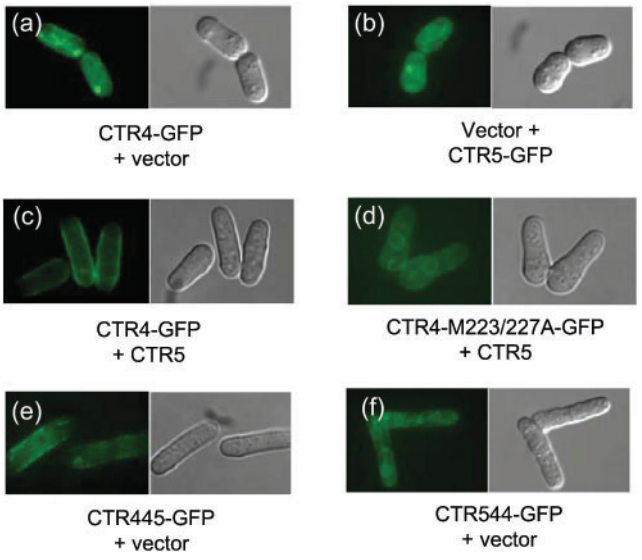


**Fig. 7.** Subcellular localization of the Ctr4 and Ctr5 protein mutants and chimeras in *Sch. pombe*. The *Sch. pombe ctr4Δctr5Δ* deletion strain was cotransformed with vector, *CTR4-GFP*, *CTR5*, *CTR5-GFP*, *CTR4-M223/227A-GFP*, *CTR445-GFP* and *CTR544-GFP*, as indicated. Transformant cells were grown and analysed as previously described (Beaudoin *et al.*, 2006). Representative images of four separate experiments are shown.

istics described for yeast and human Ctr1 may be applicable to other Ctr1-like proteins, including the *Sch. pombe* Ctr4 and Ctr5 proteins, we cannot rule out the possibility that each transporter has developed slightly different solutions to accommodate the requirements for assembly and Cu transport. Further studies will be required to decipher the characteristics of the Ctr4–Ctr5 Cu transport system in *Sch. pombe*, and will likely provide insights into our understanding of the Cu-acquisition mechanisms in eukaryotes.

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